Optical and Magnetic Resonance Studies of Formate Binding to Horse Liver Catalase and Sperm Whale Myoglobin†

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ABSTRACT: The binding of formate ion, a substrate for the peroxidatic reaction of catalase, has been investigated by magnetic resonance techniques. Comparative studies of formate binding to ferric myoglobin have also been performed. The nuclear magnetic relaxation (NMR) rate of formate and water protons is enhanced by the presence of ferric horse liver catalase. The enhancement is not changed significantly by the addition of cyanide, indicating that water and formate are still bound in the presence of cyanide. Formate proton to heme iron distances determined by magnetic resonance techniques indicate that formate does not directly bind to the heme iron of catalase or myoglobin but to the globin, and NMR relaxation occurs as a result of outer-sphere mechanisms. Evidence that water forms an inner-sphere complex with the iron atom of the catalase heme is presented. In similar experiments with ferric myoglobin, the addition of cyanide caused a large decrease in the enhancement of the proton relaxation rate of both formate and water, indicating the displacement of water and formate from the heme and the vicinity of the heme, respectively. Broad, high-spin, ferric ion electron paramagnetic resonance absorptions of catalase and myoglobin at room temperature obtained in the presence and absence of formate show that formate does not alter appreciably the heme environment of catalase or myoglobin or the spin state of the heme iron. Studies on the binding of formate to catalase as monitored by changes in the heme absorption spectrum in the visible region show one-to-one stoichiometry with heme concentration. However, the small changes observed in the visible region of the optical spectrum on addition of formate ion are attributed to a secondary effect of formate on the heme environment, rather than direct binding of formate to the heme moiety.

Catalase promotes the decomposition of hydrogen peroxide by either of two reactions, the catalatic or peroxidatic reactions:

catalatic reaction

$$\text{Cat} + H_2O_2 \rightleftharpoons \text{Cat}\cdot H_2O_2 \text{ (compound I)}$$

$$\text{C-I} + H_2O_2 \rightarrow \text{Cat} + 2H_2O + O_2$$

peroxidatic reaction

$$\text{Cat} + H_2O_2 \rightleftharpoons \text{C-I}$$

$$\text{C-I} + DH_2 \rightarrow \text{Cat} + 2H_2O + D$$

where D and DH_2 are the hydrogen donor in the oxidized and reduced form, respectively. Various organic acids and alcohols can serve as the substrate, DH_2 (e.g., formic acid, acetic acid, methanol, and ethanol), for the peroxidatic reaction. The equilibrium properties of catalase–donor complexes (i.e., the pH dependences of the dissociation constants and spectra of the various donor complexes) in the absence of hydrogen peroxide have been studied by Chance (1952).

The purpose of the present work is to elucidate the structure of a catalase–donor complex, the formate complex, by magnetic resonance techniques. Because the structure and ligand binding properties of myoglobin have been extensively studied, a comparative study of the ligand binding properties of formate to ferrimyoglobin was made. This presentation concerns the spatial relationship of the donor molecule, formate ion, and the ligands, water and cyanide, to the heme iron.

Methods

Preparation of Horse Liver Catalase. Fresh horse liver was homogenized in a Waring Blendor, followed by centrifugation of the homogenate, precipitation of catalse from the supernatant, and extraction of the catalase from the precipitate using a procedure similar to that described by Price et al. (1962). The resultant solution after extraction was dialyzed against 25% ethanol, 0.1 *M* NaCl, and 0.1 *M* acetate buffer (pH 4.7). Centrifugation of the dialysate first at +5° yielded a white pellet. Centrifugation of the supernatant at −10° yielded a pellet containing most of the catalase. The pellet was dialyzed exhaustively against 5 m*M* phosphate buffer (pH 7.8) and chromatographed on Whatman DE-52 DEAE-cellulose followed by rechromatography of the catalase fraction on DEAE-cellulose. The highest ratio of the absorbance at 405 nm to that at 275 nm, $D = 405/275$, was 0.96 and was routinely greater than 0.90. On standing for 1 month at 4°, a catalase solution in 10 m*M* phosphate buffer (pH 7.8), heme concentration = 240 μM, and $D = 405/275 = 0.96$, crystallized yielding highly birefringent long needles which when subjected to polyacrylamide disc gel electrophoresis showed only one band.

The electron paramagnetic resonance (EPR) spectrum of catalase at liquid helium temperature is shown in Figure 1A and is similar to that obtained by Torii and Ogura (1969) at liquid nitrogen temperature. The low temperature EPR spectrum shows the presence of other paramagnetic species. In addition to the signal for bile pigment, low-spin catalase

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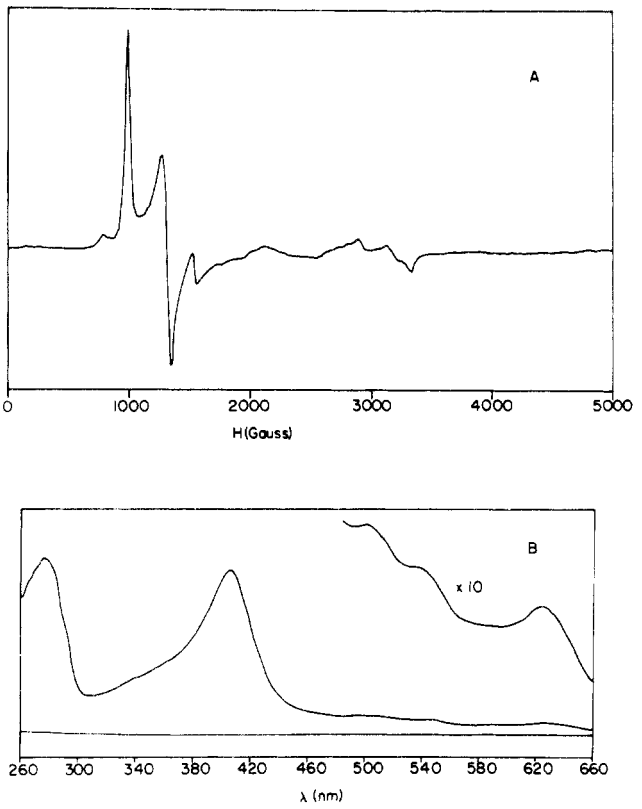

FIGURE 1: (A) X-band EPR spectrum of catalase at 4.5 K in the presence of 0.1 *M* formate (pH 7.0). (B) Optical spectrum of catalase at 20° in 20 m*M* phosphate buffer (pH 7.0).

could be present. Because it is not possible to recover native catalase from frozen solutions, another possible contribution to the low temperature EPR spectrum could arise from freezing artifacts during sample preparation. The resonance at $g \simeq 4.3$ has been ascribed to bile pigment (Torii and Ogura, 1969).

Preparation of Sperm Whale Myoglobin. The myoglobin used in this study was a generous gift of Dr. M. Tamura and was purified by the method of Yamazaki et al. (1964) for oxymyoglobin. This entails chromatography on DEAE-cellulose, followed by two successive crystallizations from ammonium sulfate.

Optical studies of the binding of formate to myoglobin and catalase were performed using either a Cary Model 118 or an Hitachi Perkin Elmer Model 323 spectrophotometer. Samples were maintained at 20 ± 0.5° using a thermoregulated water bath.

Nuclear magnetic resonance (NMR) samples were prepared by dissolving in water either catalase or myoglobin, adjusting to the desired pH, and dialyzing exhaustively against water. Samples were concentrated using a Schleicher and Schuell collodian bag apparatus. Exchange of H_2O for D_2O was done in a lucite dialysis block in which 1.0 ml of protein solution was dialyzed against 5.0 ml of buffer (see Experimental Procedures) prepared in D_2O. pD was used for protein solutions in D_2O, where pD = pH + 0.4 and pH is the value obtained using a combination electrode. Heme concentrations were determined by the reduced-alkali pyridine hemochromogen method using ϵ_{557} 34.4 (Paul et al., 1963).

NMR measurements were performed on a Varian HR-220 instrument equipped with a Fourier transform accessory. In all NMR and EPR measurements, the temperature was regulated to within ±0.5° of the desired temperature using a Varian-4257 temperature controller. Longitudinal relaxation times, T_1's, were measured using the pulse sequence of McDonald and Leigh (1973) on spinning samples. Transverse relaxation times, T_2's, were measured using the pulse sequence of Carr and Purcell (1954) on nonspinning samples. In addition to T_1 measurements at 220 MHz, T_1's of the water protons in pure water were measured at 8.13, 15.0, 24.3, and 40.0 MHz by the 180-90° null method of Carr and Purcell (1954), using an instrument described previously (Cohn and Leigh, 1962).

EPR measurements were performed using a Varian E-3 spectrometer equipped with a Varian-1024 computer of average transients. The computer was used for base-line corrections which were necessary at high gain. EPR measurements at liquid helium temperatures were made on a Varian-4502 spectrometer using an Air Products and Chemicals Heli-Tran™ Model LTD-3-110 liquid helium transfer system.

Experimental Procedures

The effect of electron-spin, nuclear-spin interaction on the longitudinal relaxation rate is described by the dipolar term of the Solomon-Bloembergen equation (Solomon, 1955; Bloembergen, 1957):

$$\frac{1}{T_{1M}} = \frac{^2\gamma_I^2 g^2 \beta^2 S(S+1)}{15r^6} \times \left[\frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{7\tau_c}{1+\omega_S^2\tau_c^2}\right] = \frac{Cf(\tau_c)}{r^6} \quad (1)$$

where γ_I is the nuclear gyromagnetic ratio, g is the spectroscopic splitting factor, β is the Bohr magneton of the electron, S is the total electron spin, r is the length of the vector between the nuclear-spin dipole and the electron-spin dipole, τ_c is the correlation time which describes the molecular events which modulate the electron-nuclear dipolar coupling, and ω_I and ω_S are the nuclear and electron Larmor precession frequencies, respectively. T_{1M} is the relaxation rate of the nucleus bound in the proximity of the paramagnetic metal ion. Equation 1 considers only the dipolar term of the Solomon-Bloembergen equation, the isotropic hyperfine term of T_1 being negligible for the following work. Equation 1, as shown, considers the case of an isotropic g tensor. Modification of eq 1 to treat the case of an axially symmetric g tensor has been made by Sternlicht (1965). The correlation time, τ_c, for the dipolar term of eq 1 is composed of three terms:

$$\frac{1}{\tau_c} = \frac{1}{\tau_r} + \frac{1}{\tau_s} + \frac{1}{\tau_M} \quad (2)$$

where τ_r is the rotational correlation time, τ_s is the electron-spin relaxation time, and τ_M is the mean residence time of the species complexed to the metal ion or the reciprocal of the first-order dissociation rate constant of the metal-ion complex. From eq 2, the shortest correlation time will contribute most significantly to τ_c. τ_s, which is in the order of 10^{-11} sec, dominates in the case of high-spin ferric hemoproteins and is about two or three orders of magnitude smaller than τ_r and about five orders of magnitude smaller than τ_M. For the case of the interaction of a proton with a spin = ½ metal ion, eq 1 reduces to:

$$r\,(\text{Å}) = 812[T_{1M}f(\tau_c)]^{1/6} \quad (3)$$

For a given species, the contribution to the observed longi-

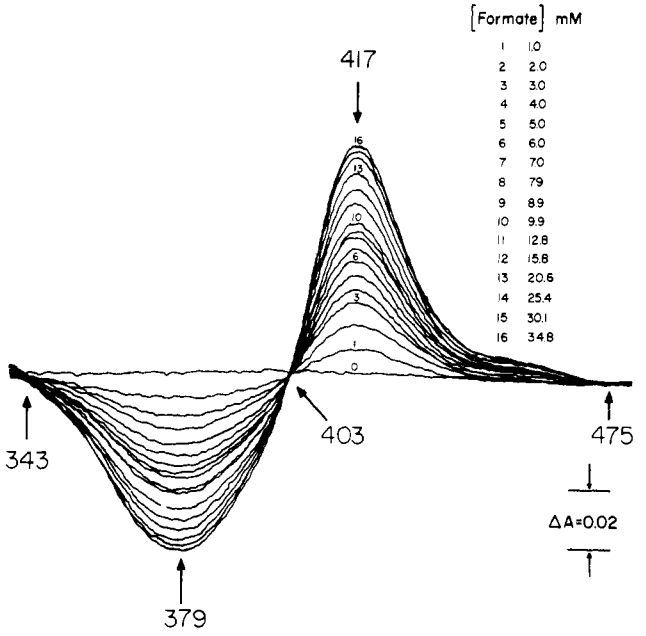

FIGURE 2: Titration of catalase with formate by optical difference spectroscopy. [Heme] = 9.8 μM, pH 7.0, 20°.

tudinal relaxation rate due to the paramagnetic ion, T_{1p}, is given by

$$\frac{1}{T_{1p}} = \frac{1}{T_{1_{obsd}}} - \frac{1}{T_{1_o}} \quad (4)$$

where T_{1_o} is the relaxation rate in the absence of the paramagnetic ion. T_{1M} is obtained from the following equation (Luz and Meiboom, 1964a):

$$\frac{1}{T_{1p}} = \frac{\alpha_M}{T_{1M} + \tau_M} \quad (5)$$

where α_M is the mole fraction of a given species complexed to the paramagnetic ion. When $\tau_M \ll T_{1M}$, $T_{1M} = \alpha_M T_{1p}$, and conditions of "fast exchange" prevail.

Hence, the calculation of the distance from formate to the heme iron atom of catalase, using eq 1, requires a measurement of (a) the dissociation constant of formate to catalase to determine α_M, (b) τ_c (measurement explained in text), and (c) T_1 of formate in the presence of catalase.

Results

The optical spectrum of catalase is shown in Figure 1B. Previous studies (Chance, 1952) of formate binding to catalase have utilized the changes in the optical spectrum of the heme group which accompany the addition of formate to determine the dissociation constant. Addition of formate to catalase effects a small change in the extinction of and a slight shift in the maximum of the Soret peak. Hence the difference spectrum of catalase plus formate[1] minus free catalase was used to determine the dissociation constants of formate from catalase and myoglobin. The results of typical titrations are presented in Figures 2 and 3. In all cases, well-defined isosbestic points were obtained. The data obtained from the difference spectra have been plotted in a

[1] At pH 7.0 and 5.0, formic acid is 100 and 91% dissociated, respectively. The measurements presented do not distinguish whether the free acid or the conjugate base binds to the protein. Because the predominant species in solution is the conjugate base, reference in the text is made to formate and not formic acid.

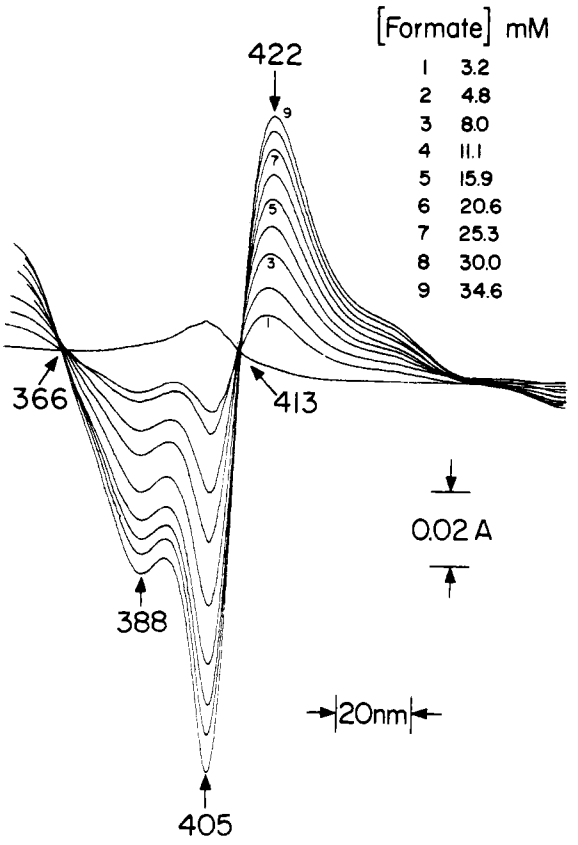

FIGURE 3: Titration of ferrimyoglobin with formate by optical difference spectroscopy. [Heme] = 2.9 μM, pH 7.0, 20°.

Scatchard plot (Scatchard, 1949) as shown in Figures 4 and 5. The number of binding sites per heme of catalase or myoglobin is approximately one in all cases. All titrations were done in duplicate and at several protein concentrations. Dissociation constants determined from the titrations are within 3% experimental error. The dissociation constants determined for ferrimyoglobin and ferric catalase are independent of the wavelength or wavelength pair used in the calculation. It has been shown that catalase has bile pigment in addition to protoheme-IX, hence the heme concentration was used rather than the protein concentration. It is evident that bile pigment does not compete with the heme for binding of formate from the observation of isosbestic points and linear Scatchard plots calculated using the heme concentration.

Table I summarizes the relaxation times of aqueous solutions of formate in the presence of ferric catalase and ferrimyoglobin. Catalase and myoglobin greatly enhance the water relaxation rate (cf. Table I, experiments 2 and 8). Since the measurements were performed in D_2O solutions, the proton relaxation rate of water was of the residual HDO. Similarly myoglobin and catalase enhance the relaxation rate of the formate proton (Table I, experiments 3 and 9). Addition of formate to 85 and 65% saturation of the formate binding site (pD 7.0) of catalase and myoglobin, respectively, causes an increase in T_1 and T_2 of water over the relaxation times in the absence of formate. Addition of cyanide to solutions of formate in the presence of myoglobin produces a dramatic increase in the formate and water relaxation times. However, the addition of cyanide to solutions containing catalase produces only a small effect on the relaxation times of formate and water protons.

Table I: Summary of Relaxation Measurements.

Expt. No.	Sample	pD	[Heme] (mM)	[Formate] (mM)	[Cyanide] (mM)	HDO T_{1obsd} (sec)	HDO T_{2obsd} (sec)	Formate T_{1obsd} (sec)	Formate T_{2obsd} (sec)	Formate T_{1M} (msec)	R(Fe–H)[b] minimum (Å)
1	Buffer	7.0	0.0	50.0	0.0	15.9		15.1			
2	Catalase	7.0	1.31	0.0	0.0	0.26	0.094				
3	Catalase	7.0	1.25	47.6	0.0	0.28	0.108	2.15	0.21	55	9
4	Catalase	7.0	1.17	47.0	6.3	0.32	0.089	2.35	0.27		
5[a]	Catalase	7.0	1.02	75.0	0.0	T_{1H_2O} = 0.12		(T_{1MH_2O} = 2.3 μsec)			2
6	Catalase	5.0	0.87	50.0	0.0	0.63	0.088	0.34	0.025	6	7
7	Catalase	5.0	0.83	47.4	10.6	0.76	0.103	0.51	0.168		
8	Myoglobin	7.0	3.00	0.0	0.0	0.78	0.26				
9	Myoglobin	7.0	3.05	50.0	0.0	1.20	0.27	1.16	0.041	49	12
10	Myoglobin	7.0	2.96	47.0	6.3	8.00	0.55	6.70	0.39		
11[a]	Myoglobin	7.0	3.07	75.0	0.0	T_{1H_2O} = 0.48		(T_{1MH_2O} = 34 μsec)			3
12	Myoglobin	5.0	3.28	50.0	0.0	1.56	0.17	0.35	0.025	20	9
13	Myoglobin	5.0	2.98	45.0	18.8	5.50	0.18	1.10	0.036		

[a] Measured at 40.0 MHz. All other measurements made at 220 MHz. [b] R(Fe–H) is the calculated distance between the iron atom and the formate proton with the exception of experiments 5 and 11 where R is the distance between the iron atom and the water proton. Measurements performed at 20°.

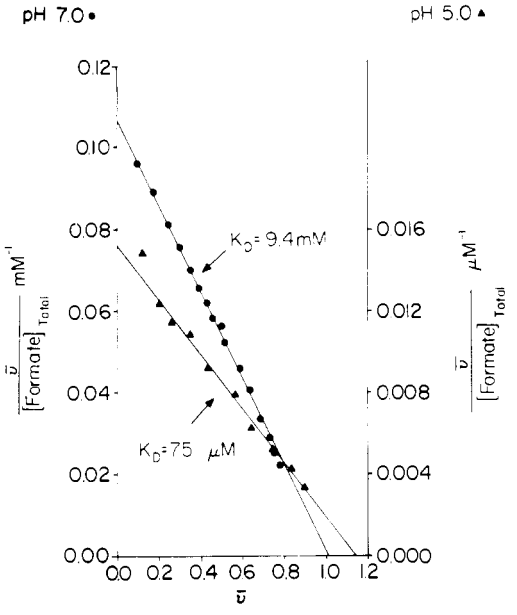

FIGURE 4: Scatchard plot of formate binding to catalase. Titration at pH 7.0 presented in Figure 3.

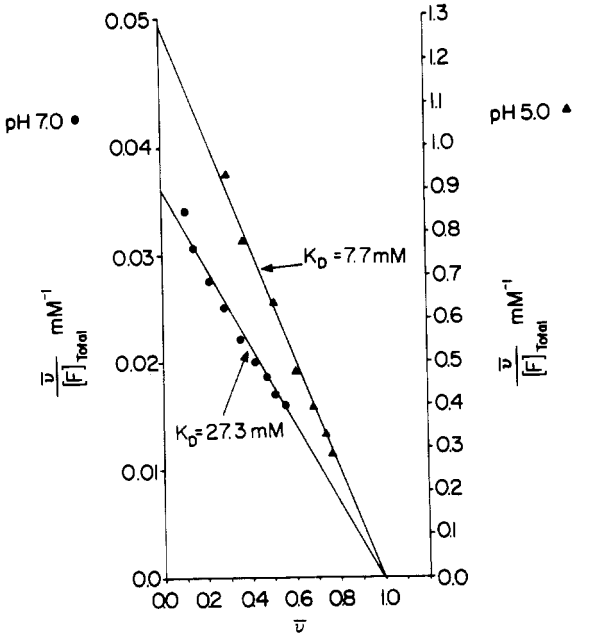

FIGURE 5: Scatchard plot of formate binding to ferrimyoglobin. Titration at pH 7.0 presented in Figure 4.

The T_1/T_2 ratios should be close to unity for ferric catalase and ferric myoglobin, which have short electron spin relaxation times. However, in a few of the experiments listed in Table I, the T_1/T_2 atios are considerably higher than expected.

Arrhenius plots of the temperature dependence of the longitudinal relaxation times are presented in Figures 6 and 7. Activation energies for the longitudinal relaxation process are 3.4 kcal/mol and approximately 1.8 kcal/mol for catalase at pD 5.0 and 7.0, respectively, and 5.7 kcal/mol for myoglobin at pD 7.0. The high ratio of the formate concentration to the heme concentration and the low value of the K_D for catalase at pD 5.0 assures that α_M will be independent of temperature under the conditions of measurement. Under similar conditions for catalase at pD 7.0, small changes in the K_D will not alter α_M significantly.

The frequency dependence of the paramagnetic contribution to T_1 of water, T_{1p}, in solutions containing catalase and myoglobin is shown in Figure 8. From eq 1, a frequency dependence of T_{1p} is observed when either $(\omega_S\tau_c)^2$ or $(\omega_I\tau_c)^2 \simeq 1$. τ_c was determined from the observed frequency dependence by an iterative procedure where τ_c was varied and the constant, C, in eq 1, was calculated. The value of τ_c which yielded the most nearly constant set of C's for the frequencies of measurement was accepted. A best fit of the observed frequency dependence is obtained when the τ_c for the formate complex of ferrimyoglobin is 3×10^{-11} sec and τ_c for the formate complex of catalase is 5×10^{-11} sec. A decrease in the τ_c to 6×10^{-12} sec is observed on the addition of cyanide to the formate complex of catalase. The observed frequency dependence in all cases is due to the dispersion in the ω_S term of eq 1. The τ_c of water bound to myoglobin and catalase was used as an approximation to the τ_c for formate bound to myoglobin and catalase. The value of τ_c obtained from the frequency dependence of T_{1p} is in reasonable agreement with the value of T_{2e} obtained

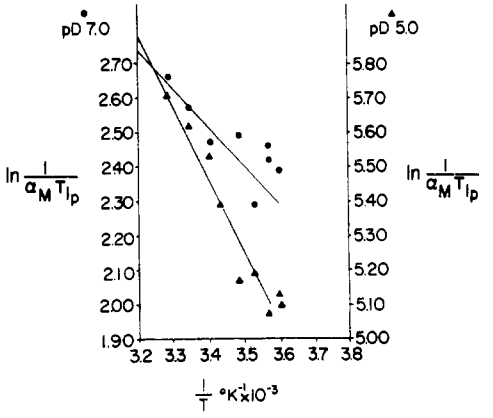

FIGURE 6: Arrhenius plots of the temperature dependence of T_{1p} of formate in the presence of catalase. [Formate] = 50 mM, [heme]$_{pD\ 7.0}$ = 0.42 mM, [heme]$_{pD\ 5.0}$ = 0.39.

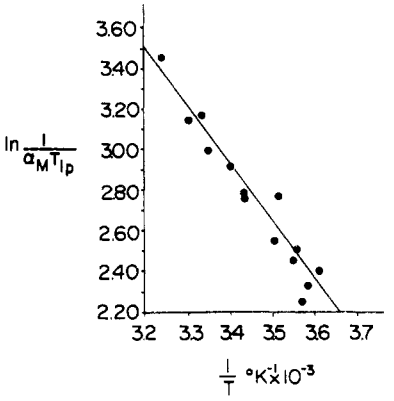

FIGURE 7: Arrhenius plot of the temperature dependence of T_{1p} of formate in the presence of ferrimyoglobin. [Heme] = 1.9 mM, [formate] = 50 mM, pD 7.0.

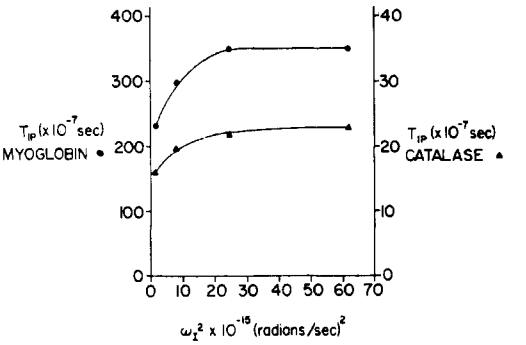

FIGURE 8: Frequency dependence of water relaxation rate in presence of ferrimyoglobin and catalase. [Heme]$_{myoglobin}$ = 3.1 mM, [heme]$_{catalase}$ = 1.0, [formate] = 75 mM, 20°.

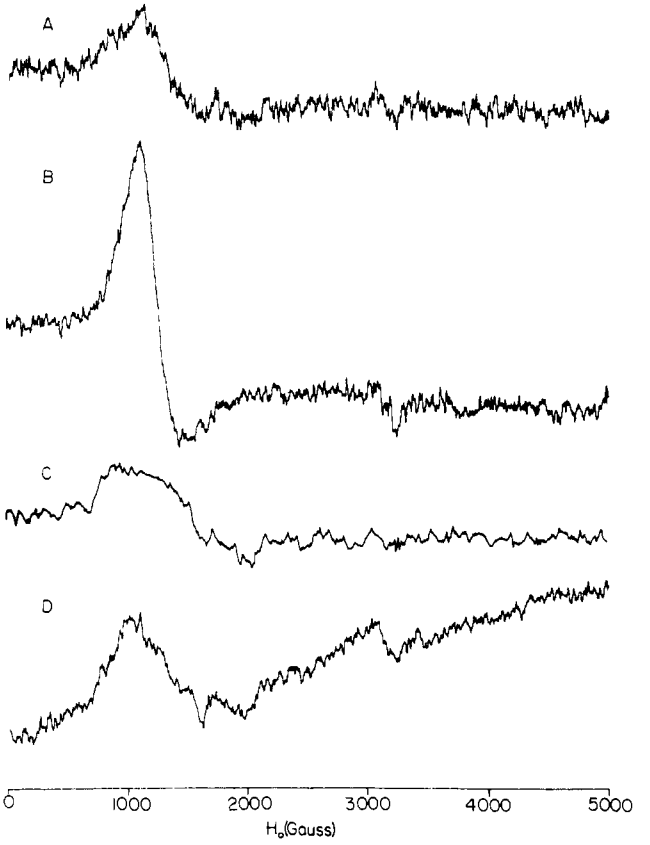

FIGURE 9: X-band EPR spectra at 20° of (A) aquo ferrimyoglobin, (B) ferrimyoglobin-formate complex, (C) aquo ferric catalase, and (D) ferric catalase-formate complex.

from the reciprocal of the line width of the EPR absorptions shown in Figure 9. High-spin ferrihemoproteins (S = 5/2) have anisotropic EPR absorptions which, at room temperature, are very broad (Asakura et al., 1972). Room temperature X-band EPR spectra are shown in Figure 9. The line widths, ΔH peak to peak, of the EPR absorptions provide estimates of the transverse electron spin relaxation time, T_{2e}, which are summarized in Table II. A narrowing of the g 6 absorption of aquo ferrimyoglobin is observed on the addition of formate which is similar to the effect of the addition of fluoride on the EPR spectrum of aquo ferrimyoglobin and aquo ferrihemoglobin observed by Asakura et al. (1972). This effect is difficult to observe on the addition of formate to ferric catalase because the line widths are approximately two times broader than those of myoglobin, although accurate values of ΔH_{pp} and the g value for catalase are difficult to measure due to the poor signal to noise (cf. Figure 9C and D).

Discussion

Changes in the optical spectrum of ferrihemoproteins accompanying the addition of potential heme ligands can reflect the coordination of the ligand to the heme iron. The measurements presented here support the conclusion that the changes of the optical spectrum of aquo ferric catalase and aquo ferrimyoglobin on the addition of formate are a secondary response to the binding of formate in the proximity of the heme prosthetic group of catalase and myoglobin. X-Ray diffraction studies show that water binds to the sixth coordination site of the heme iron of acid ferrimyoglobin and the iron to oxygen distance is about 2.1 Å (Kendrew et al., 1960). NMR studies also show that water is bound to the sixth coordination site of the heme iron of ferrimyoglobin (Fabry et al., 1971) and in the present studies to the heme iron of catalase.

The observed relaxation times of water in the presence of ferrimyoglobin and catalase are composed of a paramagnetic contribution, T_{1p}, due to the iron in addition to a contribution from the enhancement of the water relaxation rate due to the globin, T_{1o}. The paramagnetic contribution to the observed relaxation time of water is composed of a contribution from the water bound to the heme iron and a contribution of the water bound in close proximity of the heme.

The effect of the addition of cyanide to aquo ferrimyoglo-

Table II: Summary of EPR Measurements.

Sample	[Heme] (mM)	[Formate] (mM)	[Cyanide] (mM)	pH	ΔH_{pp} (gauss)	T_{2e}[a] (sec)	τ_c[b] (sec)
Catalase	2.0	0	0	7.0	(925)	(3×10^{-11})	
Catalase	2.0	100	0	7.0	(863)	(4×10^{-11})	
Catalase	1.0	75	0	7.0			5×10^{-11}
Catalase	1.3	75	5	7.0			6×10^{-12}
Catalase	2.0	100	0	5.0	(563)	(3×10^{-11})	
Myoglobin	8	0	0	7.0	463	6×10^{-11}	
Myoglobin	8	100	0	7.0	400	7×10^{-11}	
Myoglobin	5.7	0	0	5.0	400	7×10^{-11}	
Myoglobin	5.7	100	0	5.0	325	8×10^{-11}	
Myoglobin	3.1	75	0	7.0			3×10^{-11}

[a] Lower limit calculated from ΔH_{pp} using the relation $T_{2e} = 1.3 \times 10^{-7}/g\Delta H_{pp}$ (Poole, 1967). [b] Calculated from frequency dependence of the water relaxation rate. Measurements performed at 20°.

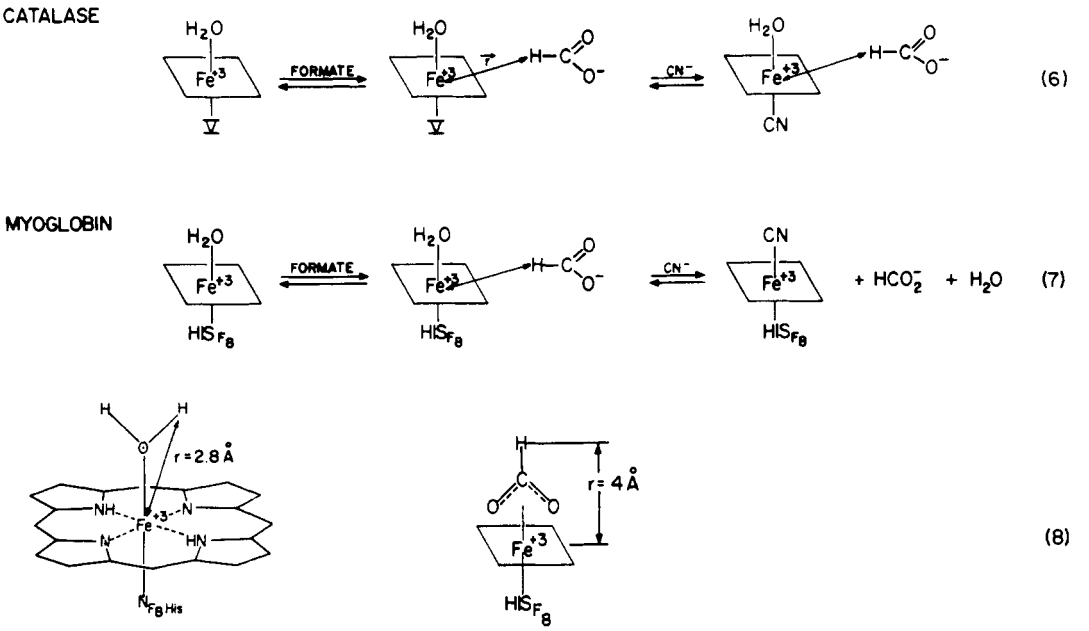

FIGURE 10: Proposed binding scheme of formate to catalase and myoglobin (eq 6 and 7). Model of direct coordination of formate and water to heme (eq 8).

bin solutions causes an increase of the T_{1p} of water because (a) cyanide displaces water bound to the sixth coordination site of the paramagnetic iron, (b) cyanide induces a high-spin to low-spin transition of the heme iron ($S = 5/2 \rightarrow S = 1/2$) resulting in a decrease in the constant C in eq 1, and (c) $f(\tau_c)$ for the interaction of water bound in the proximity of the cyano heme decreases because the electron spin relaxation time for low-spin iron is less than that for high-spin iron. Fabry et al. (1971) have used the ferrous oxy form of myoglobin ($S = 0$) to measure the contribution to T_{1obsd} of water due to the globin which was shown to be experimentally equal to the T_{1obsd} of cyanoferrimyoglobin. The equality of the relaxation time of water in the presence of cyanoferrimyoglobin and ferrooxymyoglobin implies the absence of binding sites for water in close proximity of the heme pocket as cyanoferrimyoglobin is still paramagnetic and should affect water remaining in close proximity of the heme iron.

Both ferrimyoglobin and catalase effect a decrease of the T_{1obsd} of water (cf. Table I). Addition of formate to 85 and 65% saturation of the formate binding site of aquo ferric catalase and aquo ferrimyoglobin (pD 7.0), respectively, causes a small increase of the T_{1obsd} and T_{2obsd} of water protons, but not to a value expected for T_{1o}. If water were displaced by formate from the first coordination sphere of the heme iron of ferrimyoglobin or ferric catalase, then the expected value of the water relaxation time would be in the order of 6 sec, the value of T_{1obsd} for water obtained in the presence of ferricyanomyoglobin. Hence, formate does not compete with water bound to the heme iron of either ferric catalase or ferrimyoglobin.

Addition of cyanide to ferrimyoglobin solutions containing formate causes a large increase in the T_1's of formate and water protons (cf. Table I). This is interpreted as the displacement of water by cyanide and the alteration of the formate binding site so that formate is also displaced. Ferric catalase also binds cyanide with a concomitant spin state transition of the heme iron as shown by magnetic susceptibility measurements (Torii et al., 1970) and EPR studies. However, in the case of catalase, there is a measurable but small increase in the proton relaxation times of both water and formate on addition of cyanide which is in contrast to the effect of cyanide on the proton relaxation times of water and formate in the presence of myoglobin. By eq 3, a decrease of about 25% in the catalase heme iron to formate distance would account for the relatively small change in

the T_1's of formate and water on conversion of aquo ferric catalase to ferric cyano catalase. The experiments imply that not only are water and formate simultaneously bound to catalase, but also cyanide is bound in the presence of both water and formate. The binding scheme for water, cyanide, and formate to catalase and myoglobin is summarized in Figure 10 (eq 6 and 7).

Iron to proton distances were calculated using eq 1, assuming an isotropic g tensor. This is not the case of either aquo ferrimyoglobin or aquo ferric catalase, which have axial and rhombic (Kotani, 1963) symmetry, respectively. Modification of eq 1 to treat the case of an axially symmetric g tensor, requires a knowledge of the angle between the principal axis of symmetry and the vector connecting the electron-spin dipole to the nuclear-spin dipole which for catalase and myoglobin is unknown. For all values of this angle, Sternlicht's equation (Sternlicht, 1965) predicts larger distances than the isotropic case. Hence, in Table I, the distances are designated R_{minimum}. For direct coordination of water or formate to the iron atom, as shown in eq 8 of Figure 10, one would expect an iron to water proton distance of 2.8 Å and an iron to formate proton distance of approximately 2.7–4.0 Å.

The iron to formate proton distances listed in Table I for both the case of myoglobin and catalase are in excess of the expected values for direct coordination of formate to the iron atom of the heme. However, the iron to water proton distances are compatible with direct binding of water to the heme iron atom.

The calculation of the distances listed in Table I assumes that $\tau_m \ll T_{1M}$. That $\tau_m \ll T_{1M}$ is inferred from the data presented in Figures 6, 7, and 8. The Arrhenius plot of the temperature dependence of $(\alpha_M T_{1p})^{-1}$ increases with increasing temperature over the temperature range permissible (0–40°). Typical activation energies expected for ligand exchange processes are about 10–15 kcal/mol (Luz and Meiboom, 1964b). The values measured for the activation energies of the T_1 process for formate in the presence of catalase are too low to reflect the temperature dependence of τ_M and probably reflect the temperature dependence of τ_s which is expected to be in the range of 1–3 kcal/mol (Luz and Meiboom, 1964a). The activation energy of the T_1 process of formate in the presence of myoglobin is larger than that for formate in the presence of catalase which could be due to a contribution from the temperature dependence of the chemical exchange process. The activation energies measured support the conclusion that T_{1M} is greater than τ_M, however, the limited range of temperature over which the experiments can be conducted does not establish this unequivocably.

While the presence of hydrogen peroxide might induce a binding site (s) not present in the ferric catalase, a binding site for formate ion on the globin of catalase in the absence of hydrogen peroxide which has one-to-one stoichiometry with the heme has been identified. In addition, it appears that there are two binding sites on the heme iron of ferric catalase both of which can be occupied by externally added ligands.

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References

Asakura, T., Reed, G. H., and Leigh, J. S., Jr. (1972), *Biochemistry 11,* 334–340.

Bloembergen, N. (1957), *J. Chem. Phys. 27,* 572–573.

Carr, H. Y., and Purcell, E. M. (1954), *Phys. Rev. 94,* 630–638.

Chance, B. (1952), *J. Biol. Chem. 194,* 483–496.

Cohn, M., and Leigh, J. S., Jr. (1962), *Nature (London) 193,* 1037–1040.

Fabry, M., and Eisenstadt, M. (1972), Abstracts, 164th National Meeting of the American Chemical Society, New York, N.Y., No. BIOL-60.

Fabry, T. L., Kim, J., Koenig, S., and Shillinger, W. E. (1971), in Probes of Structure and Function of Macromolecules and Membranes, Vol. 2, Chance, B., Yonetani, T., and Mildvan, A. S., Ed., New York, N.Y., Academic Press, p 331.

Kendrew, J. C., Dickerson, R. E., Strandberg, B. E., Hart, R. G., Davies, D. R., Phillips, D. C., and Shore, V. C. (1960), *Nature (London) 185,* 422–427.

Kotani, M. (1963), *Rev. Mod. Phys. 35,* 717–718.

Luz, A., and Meiboom, S. (1964a), *J. Chem. Phys. 40,* 2686–2692.

Luz, A., and Meiboom, S. (1964b), *J. Chem. Phys. 40,* 1058–1066.

McDonald, G., and Leigh, J. S., Jr. (1973), *J. Magn. Reson. 9,* 358–362.

Paul, K. G., Theorell, H., and Åkeson, A. (1963), *Acta Chem. Scand. 7,* 1284–1287.

Poole, C. P. (1967), Electron Spin Resonance, New York, N.Y., Wiley-Interscience, p 709.

Price, V. E., Sterling, W. R., Tarantala, V. A., and Recheigl, M. (1962), *J. Biol. Chem. 237,* 3468.

Scatchard, G. (1949), *Ann. N.Y. Acad. Sci. 51,* 660–681.

Solomon, I. (1955), *J. Chem. Phys. 99,* 559–565.

Sternlicht, H. (1965), *J. Chem. Phys. 42,* 2250–2251.

Torii, K., Iizuka, T., and Ogura, Y. (1970), *J. Biochem. (Tokyo) 68,* 837–841.

Yamazaki, I., Yokota, K., and Shikama, K. (1964), *J. Biol. Chem. 239,* 4151–4154.